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Functional requirement of gp130-mediated signaling for growth and survival of mouse primordial germ cells in vitro and derivation of embryonic germ (EG) cells

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SUMMARY

Leukemia inhibitory factor (LIF) is a cytokine known to influence proliferation and/or survival of mouse primordial germ cells (PGC) in culture. The receptor complex for LIF comprises LIF-binding subunit and non-binding signal transducer, gp130. The gp130 was originally identified as a signal-transducing subunit of interleukin (IL)-6 and later also found to be a functional component of receptor complexes for other LIF-related cytokines (oncostatin M [OSM], ciliary neurotrophic factor [CNTF] and IL-11). In this study, we have analyzed the functional role of gp130-mediated signaling in PGC growth in vitro. OSM was able to fully substitute for LIF; both cytokines promoted the proliferation of migratory PGC (mPGC) and enhanced the viability of postmigratory (colonizing) PGC (cPGC) when cultured on Sl/Sl[4]-m220 cells. Interestingly, IL-11 stimulated mPGC growth comparable to LIF and OSM, but did not affect cPGC survival. IL-6 and CNTF did not affect PGC. In addition, a combination of IL-6 and soluble IL-6 binding subunit (sIL-6R), which is known to activate intracellular signaling via gp130, fully reproduced the LIF action on PGC. Both in the presence and absence of LIF, addition of neutralizing antibody against gp130 in culture remarkably blocked cPGC survival. These results suggest a pivotal role of gp130 in PGC development, especially that it is indispensable for cPGC survival as comparable to the c-KIT-mediated action. We have further demonstrated that a combination of LIF with forskolin or retinoic acid, a potent mitogen for PGC, supported the proliferation of PGC, leading to propagation of the embryonic stem cell-like cells, termed embryonic germ (EG) cells. Since EG cells were also obtained by using OSM or the IL-6/sIL-6R complex in place of LIF, a significant contribution of gp130-mediated signaling in EG cell formation was further suggested.

Key words: mouse, PGC, gp130, LIF, germ cell, primordial germ cell

INTRODUCTION

Primordial germ cells (PGC) are progenitors of gametes and appear in the extraembryonic tissue at early stages of embryo (reviewed in Wylie and Heasman, 1993; McCarrey, 1993; McLaren, 1994). In mice, it is thought that PGC originate in the epiblast of the gastrulating embryo and can be first identified in the extraembryonic mesoderm at 7.0-7.5 days post coitum (dpc). PGC then enter into the embryo proper, migrate along the endoderm and mesentery of the hind gut, and colonize the genital ridges (future gonads) by 11.5 dpc. Although PGC actively proliferate during the migratory phase, they then lose their motile phenotype and enter into mitotic arrest in testis or meiosis in ovary. The precise molecular mechanisms regulating the development of PGC are still not fully understood.

PGC of the migratory phase, isolated from 8.5-10.5 dpc embryos, can be grown for several days in culture, when suitable feeder layers are provided (STO cells, Donovan et al., 1986; TM_4 cells, De Felici and Dolci, 1991; Sl/Sl[4]-m220 cells, Matsui et al., 1991). By using such in vitro culture systems, several growth factors have been shown to affect PGC (reviewed in Wylie and Heasman, 1993; De Felici et al., 1992; De Felici and Pesce, 1994). For example, several groups have clearly shown that *Steel* factor (SLF; also known as stem cell factor [SCF], *Kit* ligand [KL] and mast cell growth factor [MGF]) plays a role in the development of murine PGC in vitro (Matsui et al., 1991; Godin et al., 1991; Dolci et al., 1991) as well as in vivo (reviewed in Besmer et al., 1993). Furthermore, we recently found that tumor necrosis factor-α (TNF-α) also stimulates PGC growth (Kawase et al., 1994). In contrast, transforming growth factor-β1 inhibits PGC proliferation but

promotes their migration (Godin and Wylie, 1991). In addition to such growth-factor-mediated mechanisms, it was also reported that cAMP (De Felici et al., 1993; Dolci et al., 1993) or retinoic acid (RA)-induced intracellular signal (Koshimizu et al., 1995) has an important role in proliferation of PGC.

Another putative molecule involved in the development of PGC is the leukemia inhibitory factor (LIF; also known as differentiating inhibitory activity [DIA]), which has pleiotropic biological activities on a variety of cell types in vitro (reviewed in Smith et al, 1992; Hilton, 1992). For PGC, LIF acts in synergy with SLF on their proliferation and/or survival (Matsui et al., 1991; De Felici and Dolci, 1991) and it has also been suggested that LIF can suppress apoptosis of PGC (Pesce et al., 1993). In the presence of basic fibroblast growth factor (bFGF), LIF causes continued proliferation of PGC, leading to derivation of the embryonic stem (ES) cell-like pluripotent cells, termed embryonic germ (EG) cells (Matsui et al., 1992; Resnick et al., 1992). However, the physiological significance of LIF on PGC development is not clear at present, since LIF alone had little effect on the PGC growth (Dolci et al., 1993) and LIF-deficient mice, generated by gene targeting, were not agametic (Stewart et al., 1992; Escary et al., 1993).

LIF-mediated signal transduction is initiated by binding to a high affinity cell surface receptor complex. This is composed of two subunits: LIF-specific binding chain (referred to here as LIF-R) and the glycoprotein gp130 (reviewed in Kishimoto et al., 1992, 1994). The gp130 was originally identified as a signal-transducing subunit of the interleukin-6 (IL-6) receptor complex (Taga et al., 1989; Hibi et al., 1990) and it is also an essential component of functional receptors for oncostatin M (OSM), ciliary neurotropic factor (CNTF) and interleukin-11 (IL-11) (Gearing et al., 1992; Davis et al., 1993; Yin et al., 1993). These cytokines are not only structurally but also functionally related to LIF. For example, LIF, OSM and CNTF have the ability to maintain the pluripotential phenotype of ES cells (Conover et al., 1993; Yoshida et al., 1994; Piquet-Pellorce et al., 1994; Nichols et al., 1994). The redundant action of LIF-related cytokines is also evident in various biological functions in a variety of cells, such as hepatocytes, myeloid leukemia cells, neuroblastoma cells, megakaryocytes, osteoclasts and plasmacytomas (reviewed in Kishimoto et al., 1992, 1994).

In this study, we compared the action of known LIF-related molecules on PGC growth in vitro and examined activation or functional blocking of gp130 in PGC in order to understand the biological significance of LIF-mediated signaling on PGC development. We have demonstrated the pivotal role of gp130-mediated transduction system in growth and survival of PGC and propagation of EG cells. Possible molecular mechanisms involved in the transformation of PGC into pluripotent stem cells are discussed.

MATERIALS AND METHODS

PGC culture

Preparation and culture of murine PGC were carried out essentially as described previously (Matsui et al., 1991, 1992; Kawase et al., 1994). Random-bred Slc:ICR mice were maintained in a controlled environment. The morning when a copulation plug was found was defined as 0.5 dpc. PGC were obtained from allantois-posterior primitive streak fragment (8.5 dpc embryo), dorsal mesentery (10.5 dpc) or genital ridges (11.5 dpc). Dispersed suspensions of PGC-containing tissues were seeded onto feeder cells (Sl/Sl^4-m220 cells), which stably produce the membrane-bound form of SLF (Toksoz et al., 1992; Matsui et al., 1991, 1992). Original stocks were kindly provided by Dr Y. Matsui (Tohoku Univ., Japan). They were treated with 5 μg/ml mitomycin C for 2 hours and plated at 2×10^5 cells per well of 24-well plates (Nunc), 1 day before use. In some experiments, purified PGC (purity, more than 80%) were seeded in Matrigel (Collaborative Research)-coated dishes, as described by Dolci et al. (1993). PGC were identified by their alkaline phosphatase (APase) activity and, in some experiments, with the expression of 4C9 antigen (Yoshinaga et al., 1991), as reported previously (Kawase et al., 1994; Koshimizu et al., 1995).

Cytokines and other additives

Human IL-6, human OSM, rat CNTF, human IL-11 and human bFGF were purchased from Boehringer Mannheim. All these recombinant factors are effective on a variety of mouse cells (manufacture's description and our unpublished observation). Recombinant mouse LIF (ESGRO) was obtained from Amrad Corporation. An extracellular, soluble form of recombinant IL-6-specific receptor component (sIL-6R) was prepared as described previously (Taga et al., 1989; Yasukawa et al., 1990). Stock solutions of forskolin (FK) and all-*trans*-RA (Sigma Chemical Co.) were prepared at 10^{-2} M in dimethyl sulfoxide (DMSO).

Antibodies

Anti-c-KIT monoclonal antibody, ACK-2, was generated by Dr S.-I. Nishikawa (Kyoto Univ., Japan) and its preparation and specificity have been already described in detail (Nishikawa et al., 1991; Ogawa et al., 1991; Lev et al., 1993). Anti-gp130 monoclonal antibody, RX-19, was prepared by immunizing a rat with purified recombinant mouse gp130 protein, and this antibody inhibits the biological activities of cytokines that utilize gp130 as a signal transducer (Saito et al., unpublished data; Pennica et al., 1995).

Establishment of PGC-derived cell lines

Establishment and maintenance of PGC-derived cell lines were carried out as reported previously (Matsui et al., 1992), with some modifications. PGC cultured for 6-7 days in the presence of FK (10 μM) and LIF (10^4 U/ml) were trypsinized and seeded on freshly prepared feeder layers. This secondary culture of PGC was carried out as for the primary one. For further subculturing, tightly packed cell colonies were picked up, dissociated, reseeded on feeder cells and maintained in the presence of LIF alone (10^3 U/ml).

Statistics

Each value was presented in mean ± s.e.m. Statistical significance between values were evaluated by Student's *t*-test.

RESULTS

Effects of gp130 activation on PGC growth

We first investigated the effects of LIF and LIF-related cytokines on PGC growth in vitro. It is well known that murine PGC of different ages show different cell kinetics when co-cultured on feeder layers: PGC of migratory phase (obtained from 8.5-10.5 dpc embryos) can be kept growing for several days, but gonadal (postmigratory) PGC (on and after 11.5 dpc) undergo a rapid depletion in culture (Donovan et al., 1986; reviewed in De Felici et al., 1992). Addition of OSM in culture prolonged survival of 10.5 dpc PGC (Fig. 1A) and slowed down the degeneration of colonized PGC isolated from 11.5 dpc genital ridges (referred to hereafter as cPGC) (Fig. 1B), as has been observed with LIF (De Felici and Dolci, 1991). At both developmental stages, the

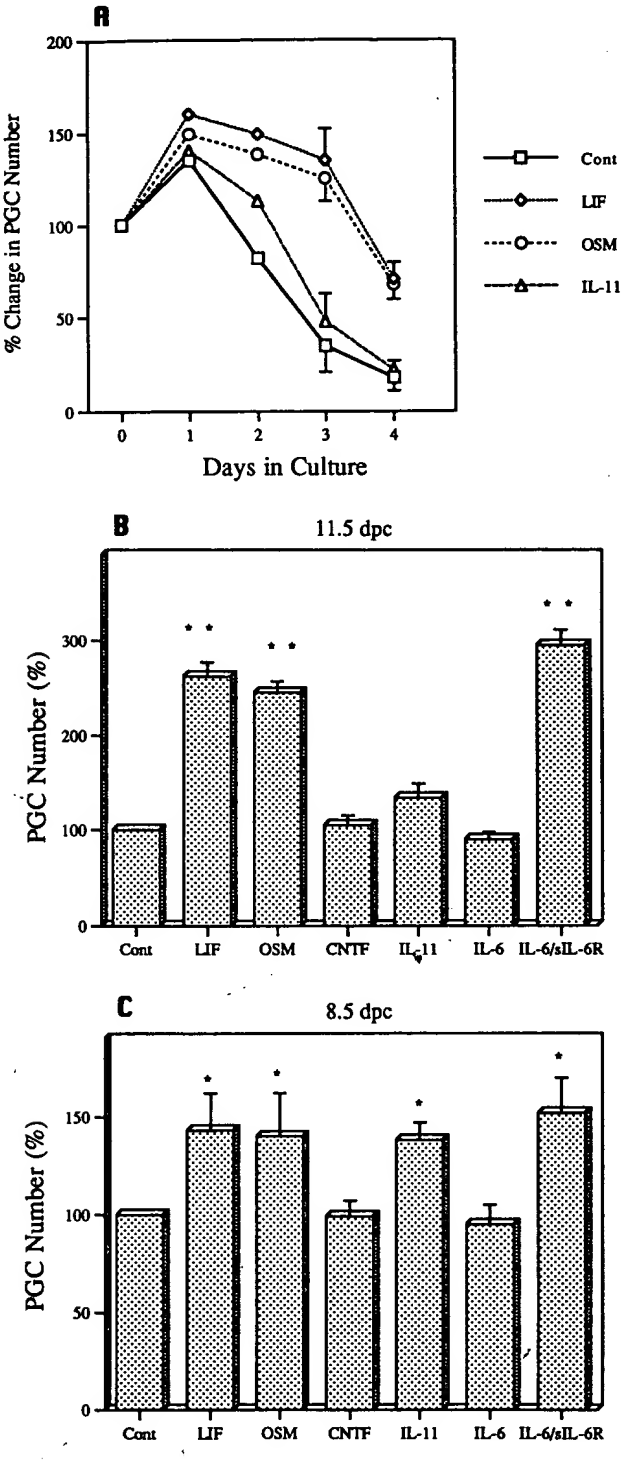

Fig. 1. Effects of LIF, LIF-related cytokines and IL-6/sIL-6R complex on PGC growth in vitro. PGC obtained from different stages of embryos were cultured on Sl/Sl^4-m220 cells. (A) Changes in the number of 10.5 dpc PGC. Initial number of PGC at the onset of culture was normalized to 100%. After 2-4 days of culture, the number of PGC in LIF- or OSM-treated group was significantly higher than that in control (P<0.05). There was no statistical difference between the value of IL-11 and that of control. The change of PGC number in IL-6- or CNTF-treated group was omitted, since both showed almost the same pattern as that in control. (B,C) Relative number of PGC obtained from 11.5 dpc (B) or 8.5 dpc (C) embryos after 2 days of culture. The number of PGC surviving in each group was counted and compared to that in controls, (as 100%). Factors were added at concentrations as follows; LIF (10^3 U [10 ng] /ml), OSM (10 U [100 ng] /ml), CNTF (100 ng/ml), IL-11 (100 U [50 ng] /ml), IL-6 (500 ng/ml) and sIL-6R (2500 ng/ml). These were optimal doses in our culture condition (data not shown). *,**Significantly different from control at P<0.05 or 0.01, respectively.

number of PGC in OSM-treated groups was comparable to that of LIF-treated groups, but other cytokines (IL-6, CNTF and IL-11) did not affect PGC number (Fig. 1A,B). Furthermore, addition of LIF or OSM significantly increased number of PGC in the migratory phase obtained from 8.5 dpc embryo (referred to hereafter as mPGC) (Fig. 1C). IL-11 also stimulated mPGC growth, but IL-6 or CNTF had no effect (Fig. 1C).

A complex of IL-6 and sIL-6R is known to bind to cell-surface gp130, induce its homodimerization and activate intracellular signaling (Taga et al., 1989; Murakami et al., 1993). The IL-6/sIL-6R complex retarded the depletion of cPGC more effectively than LIF (Fig. 1B). Moreover, the proliferative response of mPGC to the IL-6/sIL-6R complex was comparable to that to LIF (Fig. 1C). The gp130 protein is widely expressed in various tissues and cells (Saito et al., 1992), and its cell surface expression was also confirmed in cPGC, gonadal somatic cells and the feeder cells used in this study (our unpublished data). Therefore, we examined whether the IL-6/sIL-6R complex acted directly on PGC or indirectly via co-existing cells, by using Matrigel culture system (Dolci et al., 1993; Koshimizu et al., 1995). Even in the absence of feeder cells, the number of surviving cPGC treated with the IL-6/sIL-6R complex was comparable to that with LIF and much higher than that with CNTF or that of controls (Table 1).

Effects of anti-gp130 blocking antibody

The functional contribution of gp130 to PGC development was further examined by using the neutralizing antibody against gp130 (RX-19). This antibody inhibits the proliferation of an IL-6-dependent hybridoma cell line MH-60 and an IL-11-dependent plasmacytoma cell line T1165. It also blocks macrophage differentiation of a myeloid leukemic cell line M1, induced by either IL-6 or LIF, and maintenance of undifferentiated phenotypes of ES cells by LIF or CNTF. Furthermore, RX-19 can stain a BAFB03-derived transfectant expressing mouse gp130, but not its parental cell line (Saito et al., unpublished data; Pennica et al., 1995).

As shown in Fig. 2A, addition of RX-19 in the absence of LIF drastically inhibited the survival of cPGC. Even in the presence of LIF, a large population of cPGC was depleted by RX-19, suggesting the indispensable role of gp130 in their survival. The blocking intensity was comparable to that of ACK-2, neutralizing antibody against SLF receptor (c-KIT) (Matsui et al., 1991; Cheng et al., 1994). Inhibitory effects of RX-19 on mPGC growth were less apparent. In the absence of LIF, there was only a small decrease in the number of the RX-19-treated PGC, although ACK-2 antibody remarkably blocked mPGC growth (Fig. 2B). Higher doses of the RX-19 antibody caused little differences in the degree of inhibition (data not shown). In the presence of LIF, however, RX-19 completely masked the enhanced proliferation of mPGC caused by LIF (Fig. 2B).

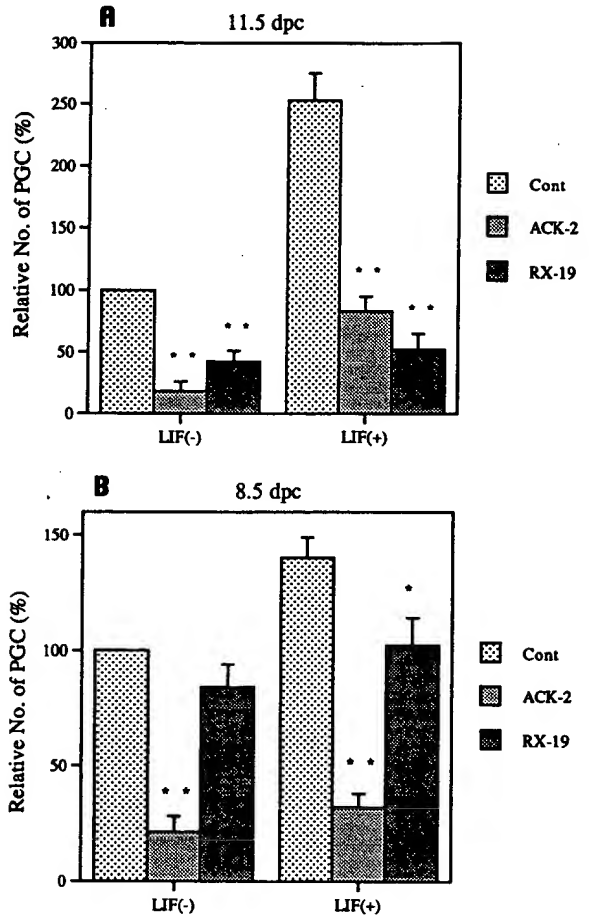

Fig. 2. Effects of neutralizing antibodies on PGC growth in culture. PGC obtained from 11.5 dpc (A) or 8.5 dpc (B) embryos were cultured on Sl/Sl^4-m220 cells for 2 days in the absence (−) or presence (+) of 10^3 U/ml of LIF. In both groups, control rat immunoglobulin (Cont), anti-c-KIT monoclonal antibody (ACK-2), or anti-gp130 monoclonal antibody (RX-19) was added at the concentration of 5 μg/ml. The number of PGC cultured in the presence of control immunoglobulin alone (LIF(−), Cont) was designated as 100%. *,**Significantly different from control at $P<0.05$ or 0.01, respectively.

Combined action of LIF with forskolin or retinoic acid

Since forskolin (FK), which increases intracellular cAMP level, or retinoic acid (RA) can also slow down the degeneration of cPGC in culture (De Felici et al., 1993; Koshimizu et al., 1995), combined action of LIF with FK or RA on the survival of cPGC was examined. As shown in Fig. 3, addition of LIF or FK alone significantly retarded the depletion of cPGC; the number of surviving PGC was higher in LIF- or FK-treated group than that in controls after 1-3 days of culture, but almost all of cPGC were depleted after 4 days. When LIF was added together with FK, the number of cPGC remaining after 3 days of culture was much higher than with LIF or FK alone. Moreover, a considerable population of PGC survived even after 5 days. The effects of RA on cPGC were essentially the same as those of FK (data not shown). In these cultures, we observed many aggregates of several PGC (Fig. 4A), which formed colonies that increased in size and became multilayered clumps (Fig. 4C). They were indistinguishable from those produced by a combination of LIF and bFGF (Fig. 4D), previously reported as EG cells (Matsui et al., 1992; Resnick et

Table 1. Effects of LIF, LIF-related cytokines and IL-6/sIL-6R complex on PGC growth in the absence of feeder layers

Treatments	No. of PGC surviving	%
Control	58.2±9.8	100
LIF	152.5±25.2*	262
OSM	132.9±14.9*	228
CNTF	86.3±10.2	148
IL-6/sIL-6R	182.2±24.8*	313

PGC were isolated from 11.5 dpc genital ridges, purified from somatic cells and seeded onto Matrigel-coated dishes. After 2 days of culture, the number of PGC surviving in each group was counted and normalized (number of PGC in control was designated as 100%). *Significantly different from control at $P<0.05$.

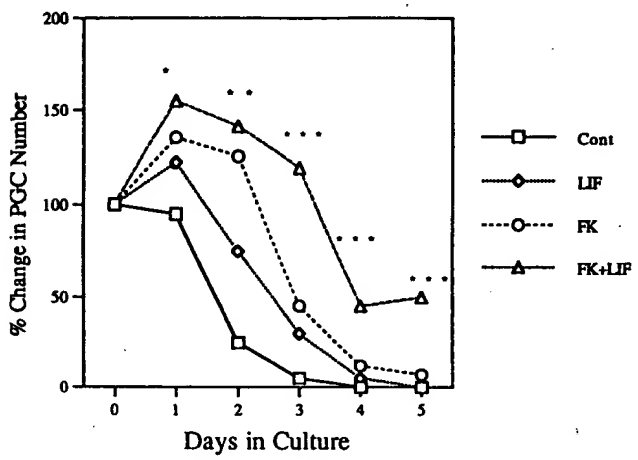

Fig. 3. Combined effects of LIF and FK on cPGC growth. PGC obtained from 11.5 dpc embryos were cultured on Sl/Sl^4-m220 cells. The initial number of PGC at the onset of culture was designated as 100%. Additives were used at concentrations as follows; LIF (10^3 U/ml) and FK (10 μM). The number of PGC in the FK plus LIF-treated group was significantly higher than that of Cont (*), Cont and LIF (**), or Cont, LIF and FK (***) at $P<0.05$.

Table 2. Number of EG cell colonies formed in the combination of several factors

	None	FK	RA
None	0	0	0
LIF	0	14.5±3.9	12.5±5.2
bFGF	0	0	0
LIF + bFGF	7.8±2.5	32.2±8.5	22.8±9.2
OSM	0	15.2±4.1	10.4±2.9
IL-6/sIL-6R	0	12.8±3.9	9.8±3.3

PGC (1/2 genital ridge of 11.5 dpc embryo) were cultured on Sl/Sl^4-m220 cells for 7 days. APase-positive colonies composed of more than 20 cells were counted. Additives were used at concentrations as follows; LIF (10^4 U/ml), bFGF (1 ng/ml), FK (10 μM), RA (2 μM), OSM (10 U), IL-6/sIL-6R (500 ng/ml and 2500 ng/ml, respectively).

al., 1992). Under the same culture conditions, we observed similar colony formation from PGC obtained from 8.5-10.5 dpc embryos (data not shown).

We further investigated the requirement of LIF and bFGF, in combination with FK or RA, for EG cell formation. In the presence of FK, RA, LIF or bFGF alone, cPGC did not produce any EG cell colonies (Table 2). Addition of LIF and RA caused EG cell formation, comparable to that in the presence of LIF and FK. The number of colonies in the presence of LIF plus FK or LIF plus RA was higher than that in LIF plus bFGF. Furthermore, a notable increase in the colony number was observed in combinations of the three factors, namely, LIF, bFGF and FK (or RA). These results indicated that bFGF and FK (or RA) were interchangeable, and additively affected the EG cell formation. However, in the absence of LIF, EG cell colonies were never formed even when bFGF and FK/RA were added. When we used the IL-6/sIL-6R complex or OSM in place of LIF, similar colonies were obtained at a comparable frequency. Furthermore, addition of the neutralizing antibody against gp130 effectively impaired colony formation in the presence of SLF, LIF, bFGF and FK (data not shown).

Lastly, we investigated whether the EG-like cell colonies formed in the absence of bFGF continue to proliferate and generate stable cell lines. In the simultaneous presence of LIF and FK, such cell colonies continued to grow through subculturing. During the first few passages, the continuous presence of LIF was required. Addition of RX-19 anti-gp130 antibody completely blocked the propagation of colonies (data not shown). So far, we have obtained several cell lines under these culture conditions (Fig. 4E), which appeared same as those obtained in the presence of LIF plus bFGF (Fig. 4F). Some of these cell lines produced cystic embryoid bodies with the outer endoderm-like cell layer. They also differentiated into fibroblast-like cells in the absence of LIF or in the presence of the RX-19 antibody.

DISCUSSION

Functional importance of gp130 signal for PGC development

Recently, Cheng et al. (1994) reported that LIF could act directly on PGC to promote their growth. They also showed that LIF-R was present on the surface of PGC and that blocking of the LIF-binding with its receptor reduced the viability of 11.5 dpc PGC in culture. However, a recent report showed that targeted disruption of the LIF-R gene did not cause defects in PGC development (Ware et al., 1995). LIF can directly bind the ligand-binding chain, LIF-R, but this low-affinity receptor cannot transduce intracellular signal by itself. Instead, LIF triggers heterodimerization between LIF-R and gp130 to form a high-affinity receptor complex (Gearing et al., 1992; Ip et al., 1992). It is well demonstrated that gp130 is not merely involved in the receptor complex formation but is essential for protein phosphorylation and signal transduction for all known cytokines related to LIF (Taga et al., 1992; Ip et al., 1992; Davis et al., 1993).

In the present study, we showed that the LIF-induced signal may be exclusively dependent on the gp130 and we confirmed the functional importance of gp130-mediated signaling in PGC development with three lines of evidence. (1) We showed that a few cytokines that can activate intracellular signaling via gp130 were able to substitute for LIF. (2) We examined the effects of IL-6/sIL-6R complex on PGC growth. The IL-6/sIL-6R complex is an useful tool to understand the signal transduction system via gp130, since IL-6/sIL-6R complex induce gp130 homodimerization and mimic the biological action of LIF and its related cytokines (Taga et al., 1989; Yasukawa et

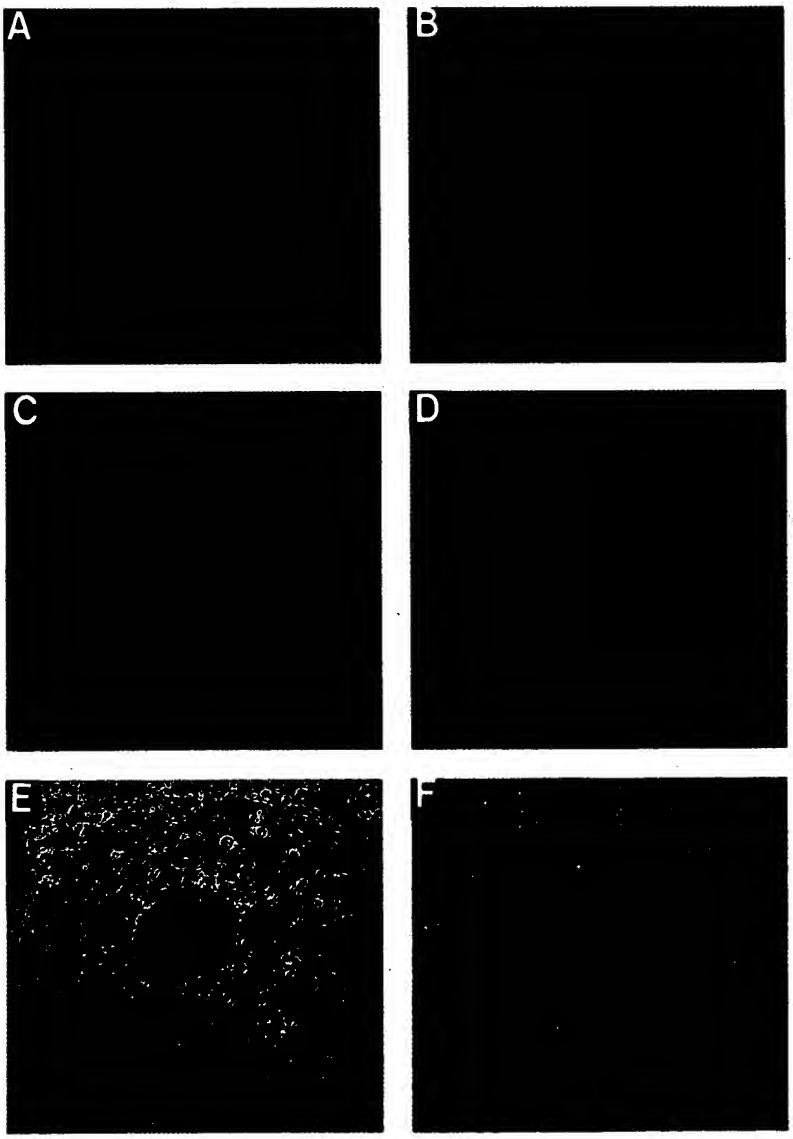

Fig. 4. Derivation of EG cells from PGC. PGC obtained from 11.5 dpc embryos were seeded onto Sl/Sl^4-m220 feeder cells and cultured in the presence of LIF and FK (A, C, E) or LIF and bFGF (B, D, F), for 4 days (A, B), 7 days (C, D), or 28 days (after three times of passages; E, F). Additives were used at concentrations as follows; LIF (10^3 U/ml), FK (10 μM) and bFGF (1 ng/ml). Cultured cells were fixed and stained for APase (red color). Scale bars indicate 200 μm (A, B) and 100 μm (C-F).

al., 1990; Yoshida et al., 1994; Nichols et al., 1994). The IL-6/sIL-6R complex was able to stimulate PGC growth and/or viability, indicating that the LIF (or other cytokines)-dependent signal transduction in PGC can be initiated via gp130 alone, but bypassing LIF-R. (3) We demonstrated that inactivation of gp130 by the neutralizing antibody completely abolished cPGC survival in vitro.

Of all cytokines examined, only OSM was able to fully reproduce the LIF action on PGC. OSM is also capable of binding the heterodimer of gp130/LIF-R complex (Gearing et al., 1992; Gearing and Bruce, 1992; Liu et al., 1992), although the presence of the OSM-specific receptor system is also suggested (Gearing and Bruce, 1992; Thoma et al., 1994). Cheng et al. (1994) showed that not only OSM but also CNTF, whose receptor complex is composed of gp130, LIF-R and CNTF-specific subunit (called CNTFRα; Davis et al., 1991), caused prolonged survival of PGC when cPGC were cultured without feeder layers. In contrast, we could not observe such effects of CNTF either in the presence or absence of feeder cells. It may be due to differences in the culture conditions, such as the medium, serum, source of CNTF, mouse strain, etc. In order to clarify this point, expression of CNTF and CNTFRα need to be examined. In our preliminary studies, we could not detect transcripts of CNTFRα in genital ridges by reverse transcriptase-polymerase chain reaction, although transcripts of gp130 and LIF-R were highly expressed (our unpublished data). We consider that OSM or a novel cytokine that binds to an unknown receptor subunit and induces gp130-activation without LIF-R, may be acting in the gp130-mediated signaling system in vivo. Very little information is available at present on the expression of LIF-related factors and their receptor subunits in PGC and their surrounding tissues, and such studies are certainly needed to elucidate the natural ligand for PGC.

It is of interest to note that IL-11 stimulated proliferation of mPGC but did not retard depletion of cPGC. Since IL-11-receptor complex is composed of gp130 and IL-11-specific subunit (Hilton et al., 1994), without LIF-R (Yin et al., 1993), insensitivity of cPGC to IL-11 may be due to the disappearance of a IL-11-specific receptor subunit in cPGC. Such a difference in the response to growth factors between developmental stages was also reported in the case of TNF-α. The mitogenic effect of TNF-α is specific for PGC at stages before and during the early migration phase, being ineffective at later stages (Kawase et al., 1994).

The functional contribution of a gp130-mediated signaling system seems to be more significant in cPGC than mPGC, as indicated previously for the effects of LIF (Matsui et al., 1991; Dolci et al., 1993; Koshimizu et al., 1995). Our neutralizing experiments in this study clearly demonstrated this difference between mPGC and cPGC. Inactivation of the SLF receptor, c-KIT, by neutralizing antibody fully impaired the development of both mPGC and cPGC, as shown previously (Matsui et al., 1991, 1992; Cheng et al., 1994). Under the same conditions, blocking of the gp130 signaling with antibody caused only small effects on mPGC growth, but it elicited rapid and complete loss of cPGC in culture both in the presence and absence of LIF.

Several lines of genetical evidence are in good accordance with our results. Murine SLF and c-KIT are encoded by *dominant white-spotting* (*W*) and *Steel* (*Sl*) loci, respectively (reviewed in Besmer et al., 1993). In mice homozygous for *W* or *Sl* mutant alleles, such as W/W^v and Sl/Sl^d, PGC cannot proliferate during migration and they fail to colonize the gonads almost completely. In contrast, apparent but less drastic reduction in PGC number was observed in mice genetically rendered deficient in gp130 by gene targeting. Whole-mount APase-staining of gonads from 11.5 or 12.5 dpc mutant embryos indicated that PGC numbers were reduced by approximately 20-60% (Yoshida, K., Taga, T., Saito, M., Kumanogoh, A., Tanaka, T., Ozono, K., Nakayama, M., Nakahata, T., Yoshida, N. and Kishimoto, T., unpublished data). Accordingly, we consider that activation of gp130 is not essential for the mPGC to survive, but it has ability to enhance their proliferation. It is also likely that the colonized mPGC are rapidly depleted in fetal gonads of mutant mice. Our present study apparently showed that gp130-mediated signaling is indispensable for survival of cPGC, probably by suppressing their apoptosis (Pesce et al., 1993) and maintaining their viability in collaboration with the c-KIT-mediated system (Matsui et al., 1991; Dolci et al., 1993; Cheng et al., 1994).

Derivation of EG cells in the absence of bFGF

We have demonstrated that the gp130-mediated signaling also has a pivotal role in the propagation of EG cells. Unexpectedly, combination of LIF with FK or RA allowed the derivation of EG cells in the absence of bFGF. EG cell lines established in this study were morphologically indistinguishable from those of the typical EG cells reported previously (Matsui et al., 1992; Resnick et al., 1992) and they had differentiation potency in vitro although their pluripotency (such as chimera formation and germ line transmission) has not been elucidated. Both FK and RA are known to act on PGC as 'mitogen', since they have ability to stimulate the proliferation of mPGC and to retard the degeneration of cPGC (De Felici et al., 1993; Dolci et al., 1993; Koshimizu et al., 1995). Originally, EG cells were reported from the culture of PGC in the presence of SLF, LIF and bFGF (Matsui et al., 1992; Resnick et al., 1992). It is not yet clear whether bFGF acts directly or indirectly on PGC. The ability of bFGF to induce production of LIF was indicated in embryonic fibroblast cell lines (Rathjen et al., 1990), but higher amounts of LIF by themselves did not produce any EG cell colonies (our unpublished data). There is a possibility that bFGF maintain or activate intracellular mechanism(s) underlying mitotic division in PGC, thereby allowing them to continue proliferation. It has been reported that bFGF stimulates PGC proliferation moderately (Resnick et al., 1992) and that ES cells are mitogenically responsive to the exogenously added bFGF (McDonald and Heath, 1994). Furthermore, it was previously demonstrated that high mitotic activity and a prolonged proliferative period of PGC correlated well with a high incidence of their transformation (teratocarcinogenesis) (Noguchi and Stevens, 1982).

It has been reported that signaling via gp130, with no essential requirement for LIF-R-mediated signaling, is also capable of propagation and maintenance of the pluripotential phenotype of ES cells (Yoshida et al., 1994; Nichols et al., 1994). An important feature in the EG cell formation is the characteristic aggregation of PGC following the formation of tightly packed clumps, which appear, like ES cells, to lack discernible cell boundaries (Matsui et al., 1992; Resnick et al., 1992). De Felici and Dolci (1991) previously reported that LIF promoted colony formation of PGC and further suggested that

the effects of LIF might be to support a subpopulation of PGC to form colonies. In addition, it is well known that gp130 activation by IL-6 stimulates multipotent colony formation in hematopoietic cells (Ikebuchi et al., 1987). Recently, it was reported that gp130 and c-KIT signalings synergize for expansion of hemopoietic progenitor cells (Sui et al., 1995). Accordingly, it may be that intracellular signaling via gp130 in combination with the c-KIT-mediated signal and altered mitotic regulation (caused by FK or RA) lead to transformation of PGC into pluripotent stem cells. It is well known that the ES, EG and embryonal carcinoma (EC) cells are similar in their appearances, pluripotency and antigenicity in spite of their different origin (reviewed in McLaren, 1992; Wylie, 1993; Wylie and Heasman, 1993). Our findings described in this report should give clues for analysis of the relationship between the PGC, EG and ES cells, which may result in more insight into the origin, determination mechanisms and differentiation of the germ cell lineage in mammals.

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